British Journal of Nutrition (2011), **105**, 1718–1723
doi:10.1017/S0007114510005544

Short Communication

Red wine prevents the postprandial increase in plasma cholesterol oxidation products: a pilot study

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(Received 24 June 2010 – Revised 23 November 2010 – Accepted 29 November 2010 – First published online 4 February 2011)

Abstract

Moderate wine consumption has been shown to lower cardiovascular risk. One of the mechanisms could involve the control of postprandial hyperlipaemia, a well-defined risk factor for atherosclerosis, reasonably by reducing the absorption of lipid oxidised species from the meal. The objective of the present study was to investigate whether wine consumption with the meal is able to reduce the postprandial increase in plasma lipid hydroperoxides and cholesterol oxidation products, in human subjects. In two different study sessions, twelve healthy volunteers consumed the same test meal rich in oxidised and oxidisable lipids (a double cheeseburger), with 300 ml of water (control) or with 300 ml of red wine (wine). The postprandial plasma concentration of cholesterol oxidation products was measured by GC–MS. The control meal induced a significant increase in the plasma concentration of lipid hydroperoxides and of two cholesterol oxidation products, 7-β-hydroxycholesterol and 7-ketocholesterol. The postprandial increase in lipid hydroperoxides and cholesterol oxidation products was fully prevented by wine when consumed with the meal. In conclusion, the present study provides evidence that consumption of wine with the meal could prevent the postprandial increase in plasma cholesterol oxidation products.

Key words: **Red wine: Postprandial oxidative stress: Oxycholesterols: Human studies**

Epidemiological studies have indicated that wine can be considered protective against CVD development when its moderate consumption is inserted in a correct lifestyle[1], including the 'instructions to drink/use', i.e. 'to be taken with meals'. A number of experimental studies have suggested that red wine compounds, especially polyphenols, might play a role in preventing the development and progression of atherosclerosis, acting through different pathways that include inhibition of lipid peroxidation, metal chelation, free-radical scavenging, inhibition of platelet aggregation, anti-inflammatory and oestrogenic activity, improvement of endothelial function, lowering of blood pressure and modulation of lipoprotein metabolism[2]. The attenuation of postprandial oxidative stress could be one of the mechanisms explaining the protective action of wine phenols[3,4]. In fact, the absorption of pro-oxidant/oxidised species with the meal can induce physiological events, such as the formation of mildly oxidised lipoprotein[5] or endothelial dysfunction[6], and inflammatory responses[7], all events linked to the development of CVD.

There is evidence that oxycholesterols are angiotoxic and could cause atherosclerosis[8]. Animal studies have shown that the addition of oxidised cholesterol to the diet increases atherosclerosis[9], and epidemiological studies have shown an association between plasma oxycholesterols and CVD[10]. Oxycholesterols have also shown to possess mutagenic and carcinogenic effects in both *in vivo* and *in vitro* studies[11].

The typical Western diet contains substantial quantities of oxidised cholesterol, and the mean dietary intake has been estimated in mg/d per person[12].

In view of the health implications of oxycholesterol absorption from food, we investigated, in a pilot study, the possibility that wine consumption with a meal influences the postprandial increase in plasma lipid hydroperoxides and oxycholesterols in humans.

Abbreviation: TMS, trimethylsilyl.

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Subjects and methods

Subjects and study design

A total of twelve volunteers (six males and six females, age 24–35 years) participated in a cross-over study. Subjects, free from known diseases, were asked to keep their diet as constant as possible during the study period, and none of them was taking any drugs or vitamin supplement. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethical Committee of the National Institute for Food and Nutrition Research. Verbal informed consent was obtained from all subjects; verbal consent was witnessed and formally recorded. Subjects ate the same test meal in two different sessions (2 weeks apart) after a 10–12 h fasting interval. The test meal, a double cheeseburger, was eaten with 300 ml of water (control) or with 300 ml of red wine (Teroldego Rotaliano, Foradori, 2004). The cheeseburger weighed approximately 200 g and contained 25·7 g of protein, 25·9 g of lipid (10·5 g SFA, 8·6 g MUFA and 0·8 g PUFA), 34·3 g of carbohydrate and 83 mg of cholesterol (US Department of Agriculture food composition table). The alcoholic grade of wine was 13·3 %, with pH 3·71, total SO_2 66 mg/l, and its phenolic content was assessed as reported by Canali *et al.*[13], with the exception of flavanols, which were estimated by LC–MS according to Mattivi *et al.*[14].

Plasma and meal analyses

Blood was collected before (time 0) and 1 and 3 h after the meal. Venous blood samples were collected into vacutainers containing opportune anticoagulants. Plasma samples were separated by centrifugation and stored at −80°C until analysis. Plasma total cholesterol, TAG and alcohol were measured by commercial kits (Futura System Srl, Formello, Roma, Italy; Sigma, St Louis, MO, USA). Plasma samples for the determination of oxycholesterols were stored at −80°C, after the addition of butylated hydroxytoluene (50 μg/ml), and analysed within 2 weeks. Total lipid hydroperoxides were measured in plasma by the ferrous ion oxidation xylenol orange-2 assay, as described by Nourooz-Zadeh[15].

Cheeseburger samples were analysed with the same methods described for plasma after homogenisation and extraction with chloroform–methanol[16].

Oxycholesterol measurement

The following four different oxycholesterols were measured in both the meal and plasma: 7-ketocholesterol (7-Keto-C), 5α,6α-epoxycholesterol, 5β,6β-epoxycholesterol and 7β-hydroxycholesterol by GC–MS[17].

The four oxycholesterols were selected because they are the most abundant in food and efficiently absorbed[18].

Briefly, plasma samples (200 μl) were added with 1 μg of the internal standard (19-hydroxycholesterol). Saponification was carried out under N_2 flow at 60°C for 90 min with 1 ml of 1 M-NaOH ethanolic solution. Samples were then extracted with cyclohexane, and the resulting organic layer was evaporated to dryness under N_2. Then, they were resuspended in 1 ml hexane and applied to solid-phase extraction (Supelclean Lc-Si cartridge; Sigma)[19]. The oxycholesterol fraction was dried under N_2 and derivatised with 70 μl of the Sylon BTZ kit (at room temperature for 45 min). GC–MS analyses were performed on an Agilent 6850A gas chromatograph coupled to a 5973N quadrupole mass-selective detector (Agilent Technologies, Palo Alto, CA, USA). Gas chromatographic separations were carried out on an Agilent HP-5MS fused silica capillary column (inner diameter 30 m × 0·25 mm and film thickness 0·25 μm). The injection mode was splitless at a temperature of 280°C. The column temperature programme was as follows: 160°C (1 min) to 280°C at a rate of 20°C/min and held for 15 min. The carrier gas was He at a constant flow of 1·0 ml/min. The spectra were obtained in electron impact mode at 70 eV ionisation energy; ion source temperature was 280°C and ion source vacuum was 10^{-5} Torr ($1{\cdot}3 \times 10^{-3}$ Pa). Analyses were performed both in total ion current and selected-ion monitoring modes. Selected-ion monitoring analyses were carried out by selecting the following representative ions: m/z 353 for the 19-OH-C trimethylsilyl (TMS) derivative; m/z 456 for the 7β-hydroxycholesterol TMS derivative; m/z 474 for the 5β,6β-epoxycholesterol TMS derivative; m/z 474 for the 5α,6α-epoxycholesterol TMS derivative; m/z 472 for the 7-Keto-C TMS derivative.

Statistical analysis

Data are presented as means and standard deviations. Statistical analysis was carried out using repeated-measures ANOVA, followed by Tukey's test for multiple comparisons. Analyses were performed with KaleidaGraph software (version 3.6; Synergy Software, Reading, PA, USA). P values $<0{\cdot}05$ were considered statistically significant.

Results

Wine composition

Total polyphenols (1871 mg/l, as catechin equivalents) were in the typical range for the variety. The concentration of total proanthocyanidins was 167·7 mg/l. The total administered dose of the major phenolics was calculated from the concentration in wine measured by HPLC at the time of the experiment. The wine had a quite high content of free anthocyanins, and the total administered dose was of 304·1 μmol. Hydroxycinnamates (85·2 μmol administered) consisted mainly of *trans*-caftaric acid, coutaric acid and *trans*-coumaric acid, with minor amounts of fertaric acid and grape reaction product (i.e. *trans*-2-S-glutathionyl-caftaric acid). Free flavanols (total of 82·9 μmol) consisted of epigallocatechin, (+)-catechin, epicatechin and gallocatechin. Myricetin was by far the main flavonol (20·5 μmol of total flavonols). Other minor phenolics were tyrosol (29·8 μmol) and the four monomers of resveratrol (for a total of 5·4 μmol).

In summary, the single dose of Teroldego wine provided 561 mg of phenolics (which is approximately in the millimolar level, assuming an average molecular weight of 500).

Lipid hydroperoxides and oxycholesterols in the test meal

The lipid hydroperoxide content of the test meal was 237 (SD 36) μmol of H_2O_2 equivalents.

As for oxycholesterols, the test meal contained 498 (SD 147) μg of 7-ketocholesterol, 138 (SD 48) μg of 5α,6α-epoxycholesterol, 91 (SD 6) μg of 7β-hydroxycholesterol and 70 (SD 6) μg of 5β,6β-epoxycholesterol.

When expressed per g of the test meal, total oxycholesterols were 3·9 (SD 1·1) μg/g, and this value is in accordance with literature data. van de Bovenkamp *et al.*[12] reported 3·6 μg of total oxycholesterols/g of a cooked mixed Dutch diet, while Baggio *et al.*[20] and Rodriguez-Estrada *et al.*[21] reported a concentration of about 2 μg/g of hamburger.

Effect of the control and wine meals on plasma lipids, lipid hydroperoxides and oxycholesterols

Plasma concentrations of total cholesterol, TAG and alcohol, before and after the control and wine meals, are shown in Table 1. As expected, there was an increase in plasma TAG after the consumption of both meals, while ethanol, as expected, increased significantly only after the wine meal. Cholesterol concentration did not change significantly after both meals.

As shown in Fig. 1, the control meal induced a significant increase in total plasma lipid hydroperoxides. On the contrary, the wine meal not only prevented this increase, but also reverted it, inducing a significant decrease in plasma lipid hydroperoxides.

Fig. 1 shows also the effect of the meal on plasma oxycholesterols. The control meal induced a significant increase in 7β-hydroxycholesterol and 7-ketocholesterol concentrations. This increase was statistically significant 1 h after the consumption of the meal. The postprandial increase in these two oxycholesterols was fully prevented when wine was consumed with the meal. Indeed, wine consumption induced a significant decrease in 7β-hydroxycholesterol. Both 5α,6α-epoxycholesterol and 5β,6β-epoxycholesterol showed the same trend as observed for 7β-hydroxycholesterol and 7-ketocholesterol, even if their postprandial changes (after both the control and wine meals) were not statistically significant.

An estimation based on the subjects' volume of plasma (55% of volume of blood, calculated individually as 7% of their body weight[22]) indicates that total plasma oxycholesterols (the sum of the measured four oxycholesterols) represented 105 (SD 29) and 95 (SD 26)% of the ingested dose 1 and 3 h after the control meal, respectively. As evident from Fig. 1, after the wine meal, total plasma oxycholesterols decreased below the baseline value (−55 (SD 28) and −31 (SD 41)% of the ingested dose, at 1 and 3 h, respectively).

Discussion

Some authors suggest that the absorption from meals of the products of lipid oxidation could be, at least partially, the link between postprandial lipaemia and atherosclerosis[23].

Oxycholesterols are a common component of the Western diet, and their presence is striking in fast food and processed food. Studies in both humans[18] and animals[24] have demonstrated that oxycholesterols are absorbed by the small intestine, transported in plasma by chylomicrons and incorporated into lipoprotein. As oxycholesterols posses several proatherogenic activities[8], a delayed clearance of these compounds from the circulation could be harmful.

Although oxysterols are principally derived from dietary sources, circulating oxycholesterols may be produced enzymatically at the intracellular level and/or from lipoprotein oxidation into the circulation[25], or by free radical-catalysed oxidation of cholesterol during digestion, both at gastric[4,26] and intestinal levels[27]. Even if oxycholesterols have a faster plasma clearance than 'normal' cholesterol, the level of oxycholesterols in plasma can remain elevated for more than 6–8 h after a meal[18]. Thus, the frequent consumption of foods rich in oxycholesterols can result in a continuous exposure during most of the day.

According to the literature, the estimates of the extent to which oxycholesterols are absorbed vary from 6 to 93%[28]. This wide range of results may be due to both the dose and vehicle used to administer the oxycholesterols[28]. Our estimate seems to indicate a complete absorption (105 (SD 29)% of the ingested dose) 1 h after the control meal. We hypothesise, however, that some of the oxycholesterols present in plasma derive from the oxidation of the cholesterol contained in the meal during the digestive process. Although the formation of oxycholesterols during digestion cannot be demonstrated by the present study design, several authors provide evidence that lipid oxidation can occur during digestion[26,27,29], and that the presence of antioxidants in the digestive tract can protect from this event[26,30].

A few animal studies have demonstrated that supplementation with antioxidants can prevent the increase in

Table 1. Plasma concentration of some metabolic parameters in plasma after a double cheeseburger meal with 300 ml of water or wine

(Mean values and standard deviations, *n* 12)

	Control						Wine					
	0		1 h		3 h		0		1 h		3 h	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Total cholesterol (mg/l)	1630	300	1650	260	1610	300	1660	250	1640	270	1660	260
TAG (mg/l)	820	160	820*	240	1070*	330	670	210	970	530	1230*	240
Alcohol (%, w/v)	0·01	0·01	0·01	0·01	0·01	0·01	0·01	0·01	0·08*	0·03	0·05*	0·02

* Mean values were significantly different from those of homologous time 0: $P<0·005$ (by repeated-measures ANOVA, followed by Tukey's test).

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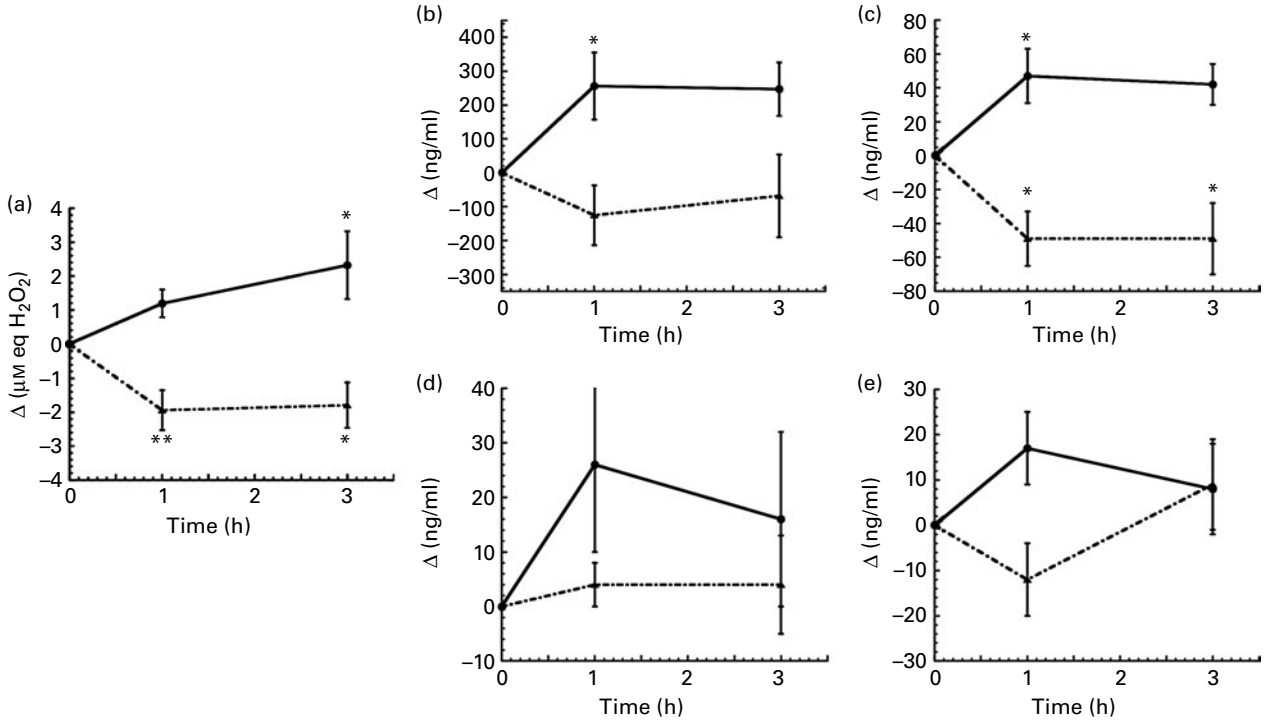

Fig. 1. Time course of plasma (a) lipid hydroperoxides and oxycholesterols ((b) 7-ketocholesterol, (c) 7-β-hydroxycholesterol, (d) 5α,6α-epoxycholesterol and (e) 5β,6β-epoxycholesterol) after the administration of the control meal (—) or wine meal (···). Values are means, with standard errors represented by vertical bars (*n* 12). Mean values were significantly different from those of homologous time 0: * $P<0{\cdot}05$ and ** $P<0{\cdot}01$ (by repeated-measures ANOVA, followed by Tukey's test).

circulating oxycholesterols induced by a high-fat diet[31], while the addition of pro-oxidant species to the diet results in a drastic increase in hepatic oxycholesterols[32]. Finally, some human studies have demonstrated that long-term supplementation of antioxidants can reduce the plasma level of oxycholesterols[33]. Thus, the composition of diet (its antioxidant/pro-oxidant balance) has a great influence on the circulating level of oxycholesterols.

However, in all these studies, the effects of antioxidants on the circulating level of oxycholesterols have been studied after a chronic supplementation with a high-fat diet. The present study, instead, demonstrates that wine could prevent the acute oxycholesterol 'toxicity' induced by a single high-fat meal.

It has been demonstrated that wine or wine polyphenols consumption can hinder many harmful postprandial events, such as oxidative stress and endothelial dysfunction. Red wine consumption with the meal reduces the susceptibility to oxidation of postprandial LDL[3] and prevents the postprandial increase in plasma lipid hydroperoxides and malondialdehyde[4]. A standardised grape product suppresses the meal-induced impairment of vascular endothelial function[34].

In the present study, we have demonstrated for the first time that a glass of wine can prevent the postprandial increase in plasma lipid hydroperoxides and oxycholesterols after the ingestion of a high-fat, high-cholesterol meal. The peak point seems to correspond to 1 h, but our experimental design (last point 3 h after the meal) cannot indicate the length of the effect; this is a limitation of the present study.

Epidemiological studies have indicated a J-shaped relationship between wine consumption and CVD risk[35]. The shape of the curve is the result of the opposite effects of wine/alcohol on the cardiovascular system: 'positive', such as an increase in HDL-cholesterol, anti-thrombotic effects, improved endothelial function, reduced insulin resistance, etc. and 'negative', such as an increase in postprandial TAG level (that is evident also from our data, see TAG in Table 1) and induction of lipid peroxidation by ethanol.

In this view, the postprandial reduction in oxycholesterols and oxidised lipids could represent a further 'positive' effect of wine. It is well known, in fact, that oxysterols are present in atherosclerotic lesions[36–38] and atherogenic lipoprotein[39], and possess several proatherogenic activities, such as cytotoxicity on endothelial and arterial smooth muscle cells, down-regulation of LDL receptors on vascular cell, proinflammatory activities (induction of cytokine release by macrophages and of the expression of adhesion molecule in endothelial cells)[40–45]. Finally, several animal studies have shown that oxycholesterols promote the onset and the development of atherosclerosis[9,46,47].

The results of the present pilot study do not allow explanation of the mechanisms/reactions by which wine counteracts the postprandial increase in circulating oxidised lipids, so that we can just speculate on some possibilities, needing experimental confirmation.

Wine polyphenols and/or alcohol could minimise the postprandial increase in plasma lipid hydroperoxides and cholesterol oxidation products by (1) reducing lipid peroxidation

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products or preventing their formation in the digestive tract[26], (2) preventing or delaying fat absorption[48–50], (3) inducing detoxifying enzymes in the gut and liver[51,52], (4) enhancing the cholesterol oxidation product clearance, through the induction of enzymes involved in the cholesterol catabolism towards bile acids[53,54] and (5) chemically reducing lipid hydroperoxide and/or oxycholesterols into the circulation after their absorption.

We studied the effect of wine as a whole, thus we cannot determine which is the wine component (alcohol or polyphenols) responsible for the observed effects and whether other forms of alcoholic beverages could have similar effects. This matter is definitely very interesting, and it should be the object of further investigation. Similarly, it should be important to study how a different ratio of wine:meal oxycholesterols could affect the wines capacity to cope with the increase in plasma oxycholesterols.

The present study provides evidence that consumption of wine with a meal could prevent and 'counterattack' the postprandial increase in plasma lipid hydroperoxides and oxycholesterols, thus protecting the organism from their potential proatherogenic effect. In this view, the controversial effect of a moderate wine consumption on 'health' (different effects *v.* different diseases) could be revised, as the modality of drinking wine (either during or separately from the meal) could represent a decisive factor.

Acknowledgements

We acknowledge the financial support by grant 'NUME' (DM 3688/7303/08) from the Italian Ministry of Agriculture, Food and Forestry. We thank all the volunteers for their participation. Kariklia Pascucci is acknowledged for her kind support in the daily laboratory work. F. N., A. M., A. R., F. M., R. M. M. and C. S. were responsible for the study design, endpoint assays, data analyses and interpretation, and writing of the manuscript. M. F. assisted in conducting of the experiments. All authors reviewed the manuscript and provided scientific and editorial input. None of the authors had a conflict of interest.

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British Journal of Nutrition